REMARKS/ARGUMENTS

Claims 1-5, 7-27, and 29-34 are pending in the above-identified application. Claims 1, 22, and 34 have been amended. Support for these amendments is identified in the following remarks. No new matter is added by these amendments.

Rejections Under 35 U.S.C. §112

Claims 5 and 15 stand rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. The rejection has been maintained for reasons of record. As previously stated, Applicants will provide a Declaration assuring public availability of any deposited material when allowable subject matter has been indicated.

Rejections Under 35 U.S.C. §103

Claims 1-4, 7-14, and 16-34 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Midthun *et al.* (*J. Virol.* 53:949-954, 1985), designated Midthun '85, Midthun *et al.* (*J. Clin. Microbiol.* 24:822-826, 1986), designated Midthun '86, Hoshino *et al.* (*J. Med. Virol.* 51:319:325, 1997), and Clark *et al.* (U.S. Patent No. 6,113,910). The Examiner has maintained the rejection for the reasons of record.

The Examiner has summarized the claims of the instant application as being primarily drawn to a multivalent immunogenic composition comprising at least four bovine strain reassortant rotaviruses and a physiologically acceptable carrier, wherein each bovine reassortant rotavirus comprises a single rotavirus VP7 gene that encodes a protein that is immunologically cross-reactive with an antigenically distinct human VP7 serotype and the

remaining 10 genes derived from the bovine UK strain, and wherein the composition induces an effective immunogenic response to each antigenically distinct human rotavirus VP7 serotype without causing a transient low level fever in a statistically significant number of vaccinees when each of the rotavirus reassortant serotype is administered at a dosage of less than $10^{6.0}$ plaque forming units.

Midthun *et al.* '85 and '86 are alleged by the Examiner as teaching four human x bovine reassortant rotaviruses where the reassortants have one human gene (D (serotype 1), DS-1 (serotype 2), P (serotype 3) and ST3 (serotype 4) from a human rotavirus serotype and where the bovine parent/backbone, which is the UK strain, provides the remaining 10 genes. The Examiner acknowledges that neither Midthun *et al.* '85 nor Midthun '86 teach a multivalent immunogenic composition of four reassortant rotaviruses, a physiologically acceptable carrier, induction of an immunogenic response without causing a low level fever, or a dosage.

However, the Examiner alleges that Clark *et al.* teach the use of WC3 strain of bovine rotavirus to produce human x bovine reassortants and the combining of different reassortant rotaviruses into a single composition. The Examiner also notes that Clark *et al.* states "[t]he vaccine compositions of the invention may desirably include other rotavirus reassortants of the invention, in addition to a G1 reassortant...For example, in one desirable formulation, the invention provides a vaccine composition containing WI79-3, 9 and WI79-4 (serotype G1 and P1, respectively). This composition has been shown to elicit a stronger immune response than does a single reassortant rotavirus containing both the human v.p.4 and the human v.p.7 (encoded by gene segment 9)....Suitable combination vaccines, which may be univalent, bivalent, trivalent, quadrivalent, quinquavalent, or sexavalent may include various combinations of the G1, G2, G3, G4, P1 and P2 reassortants. Other suitable reassortants of the invention may be selected for use in vaccine compositions other than those specified in Table 2 by reference to Table 1 above and the present specification." Clark *et al.* is also alleged by the Examiner to teach suitable carriers, liquid dose forms, buffers, lyophilized forms, adjuvants, multiple administrations, and methods for stimulating the immune system. The Examiner also alleges

that Clark *et al.* teach a general dose range between 10^6 and 10^9 and other dosages of $10^{5.5}$, $10^{6.5}$ and $10^{7.5}$.

Hoshino *et al.* is alleged by the Examiner to teach that the four human serotypes (serotypes 1-4, also disclosed in Midthun *et al.* '85 and '86) are the most epidemiologically important serotypes.

The Examiner alleges that it would have been obvious to one of ordinary skill in the art to modify the teachings of Midthun *et al.* '85 and '86 to produce a multivalent composition with two, three, four, five, six, etc. reassortants. One would have been motivated to do so given the numerous teachings of Clark *et al.*, in particular, the teaching to produce a multivalent composition of reassortants and to include more than one reassortant to elicit a stronger immune response (see col. 7 lines 43-49) and the teachings of Hoshino *et al.* There would have been a reasonable expectation of success given the knowledge that Clark *et al.* successfully vaccinated subjects with reassortant vaccines (see Examples) and also given the knowledge that WC3 strain of Clark *et al.* and the UK strain are of the same serotype (serotype 6). Finally, the prior art reference (or references when combined) teaches or suggests all the claim limitations. Thus, the invention as a whole was clearly *prima facie* obvious to one of ordinary skill in the art at the time the invention was made. In addition, the Examiner alleges that as for the number of components in the vaccine composition and dosages claimed, it is well within the purview of one of ordinary skill in the art to optimize dosages recited in the claims.

Applicants strongly disagree with the Examiner's interpretation of the art, but in order to further expedite prosecution of certain subject matter disclosed in the instant application have amended claims 1, 22 and 34 to set forth the present invention with greater particularity. In particular, claims 1, 22 and 34 have been amended to recite that the compositions and methods stimulate "the immune system of an infant of less than six months of age to produce an effective immunogenic response to human rotavirus VP7 serotype antigen without significant transient low level fever in a statistically significant number of vaccinees." Support for this amendment

can be found in the specification as filed at, for example, Example 1 beginning on page 13, Table 1, page 20, and in Example 2 beginning at page 28.

In regard to Clark *et al.* successfully vaccinating subjects with reassortant vaccines, no such disclosure is provided for an effective bovine reassortant composition wherein each reassortant rotavirus comprises a VP7 antigen immunologically cross-reactive with a human VP7 serotype and the remaining genes from a bovine rotavirus at a dosage of less than 10^6 pfu. In particular, there is no disclosure of a bovine UK rotavirus x human reassortant at any concentration. In Example 4, Clark discloses the administration of a composition comprising a human x bovine WC reassortant wherein gene 3 and gene 9 (vp7) are from a human rotavirus and the remaining genes are from the bovine rotavirus strain WC3. The composition was administered at various concentrations of $10^{5.5}$ pfu, $10^{6.5}$ pfu, and $10^{7.5}$ pfu. At lines 58 through 64, Clark *et al.* state "30 of 54 infants, or 57%, given any dose of vaccine developed a virus-neutralizing serum antibody response to one or more of rotavirus serotypes G1, G3 or bovine. This immune response to a primary dose of WI79-3,9 was most often directed against the bovine serotype of rotavirus, WC3, or serotype G1, WI79, reflecting the dual antigenic constitution of the rotavirus reassortant." As such, Clark *et al.* have no support for single gene substitution of the VP7 gene. Further, this statement is entirely ambiguous as to the effectiveness of the WC3 rotavirus reassortant composition provided at a dosage of $10^{5.5}$ pfu. Further, there is no indication as to the actual age of the "infants" in the study. Still further, only two infants were administered the $10^{5.5}$ pfu dosage of WC3 reassortant rotavirus, a total of 3.7% of the vaccinees. As such, the skilled artisan can draw no conclusion regarding the effectiveness of the human x WC3 bovine VP7 reassortant vaccine composition at a dosage $10^{5.5}$ pfu, much less a single substitution VP7 serotype bovine UK rotavirus reassortant composition.

In further support of this conclusion, Applicants attach hereto a copy of Clark *et al., Vaccine* 8:327-332, 1990. The study reported in this article appears to be the same as that disclosed in the Clark patent. In particular, Applicants respectfully wish to draw the Examiner's attention to the study described in Table 3 and discussed beginning in the left column of page

329. In this study as in the Clark patent, 54 total vaccinees are described wherein 2 "infants" are administered a dosage of $10^{5.5}$ pfu of reassortant rotavirus WI79-9 (also known as the double gene reassortant WI79-3,9 as set forth in the Clark patent). The "infants" were both 12 months of age. It is well known to those artisans skilled in the rotavirus art that it is easier to induce an effective immunogenic response in a 12 month old infant than in an infant of less than six months of age. In part this is due to the presence of maternal antibodies that reduce the effectiveness of any rotavirus composition in younger infants. In addition, older infants have typically been exposed to rotavirus wherein an immune response has been developed and therefore much of the response to the immunization in these infants is due to a more easily induced memory response. Therefore, there is no disclosure that suggests or describes an effective dosage of any human x bovine reassortant at less than 10^6 pfu, much less an effective composition comprising a bovine UK reassortant rotavirus. The effectiveness of a multivalent bovine UK x human rotavirus VP7 reassortant at a dosage of less than 10^6 pfu was not just routine optimization, but was an unexpected result to the skilled artisan. No other bovine rotavirus strain reassortant had previously been found to be effective at a dosage of less than 10^6 pfu and typically were not found to be effective at dosages of less than 10^7 pfu. The ability to use UK x human VP7 reassortant rotavirus compositions at such a low dosage provides a commercial advantage in that less virus is required and in many cases a concentration step from viral culture is not required.

Further, the Examiner has suggested that the immunogenicity of the bovine rotavirus WC3 and UK strains has some relationship to the serotype of the bovine rotavirus. Both bovine rotavirus strains WC3 and UK are serotype 6. Serotype 6 refers to the immunological relationship between the VP7 antigens which have been replaced by the human VP7 gene in the human x bovine reassortant rotavirus. Effective immunogenicity of the reassortant rotavirus is related in part to the reactivity of the various protein products of the virus and also to the ability of the reassortant rotavirus to replicate in the vaccinee. As such, the strength of the immune response has no relationship to the serotype of the WC3 and UK rotavirus strains.

Given that no multivalent immunogenic composition comprising at least four bovine strain reassortant rotaviruses and a physiologically acceptable carrier, wherein each bovine reassortant rotavirus comprises a single rotavirus VP7 gene that encodes a protein that is immunologically cross-reactive with an antigenically distinct human VP7 serotype and the remaining 10 genes derived from the bovine UK strain, and wherein the composition induces an effective immunogenic response to each antigenically distinct human rotavirus VP7 serotype in infants of less than six months of age, no particular parameter has be recognized as a result-effective variable. As such, the determination of the optimum or workable ranges of any variable can be characterized as routine experimentation and the present invention provides an unexpected result from the prior art.

The Midthun *et al.* references as presented previously add nothing to the teaching of Clark *et al.* to suggest that a human x bovine UK reassortant could have been successfully used at a dosage of less than 10^6. Further, Hoshino *et al.* merely disclose that certain serotypes of rotavirus are of clinical importance, but does not suggest or disclose either alone or in any combination with the Midthun *et al.* references or Clark *et al.* that a multivalent composition comprising less than 10^6 pfu of each bovine UK x human VP7 single gene substitution reassortant rotavirus would be immunogenically effective in infants of less than six months of age. The result of such a low concentration of reassortant UK x human rotavirus providing an effective immunogenic composition in infants of less than 6 months of age was unexpected.

Still further, the Examiner has alleged that the disclosure in Midthun *et al.* 1985 that it was "likely that the presence of 10 animal rotavirus genes in these reassortants will render such viruses attenuated for humans. This latter supposition is supported by the fact that bovine rotavirus UK and RRV have been administered to susceptible volunteers with a low level of serum antibodies and did not produce illness (Kapikian *et al.*, in press; Wyatt *et al.*, in press)" suggest that the UK strain be successfully used in human x bovine reassortants at a dosage of less than 10^6. Applicants disagree with this characterization of the art. At most Midthun *et al.* 1985 provides the suggestion that a composition comprising the reassortant bovine x human

reassortant rotavirus might be useful to induce an effective immune response in an adult, but provides no expectation, much less a reasonable expectation, that the composition would be either attenuated or induce an effective immune response in either a child or an infant. As well known to an artisan skilled in the rotavirus art adults respond differently to rotavirus than do children and children respond differently than infants. It is also well known that infants of less than six months of age react differently than adults, children and older infants (6 to 12 months of age). As above, younger infants (less than 6 months of age) have maternal antibodies to rotavirus that can reduce the effectiveness of any rotavirus composition that had been used successfully in young children or older infants (6 to 12 months of age). As such, there is no expectation that a bovine UK composition would be appropriately attenuated or immunogenically effective in children or infants, much less a composition comprising bovine UK x human VP7 reassortant rotavirus.

Applicants respectfully request that the Examiner reconsider and withdraw the rejection of claims 1-4, 7-14 and 16-34 under 35 U.S.C. § 103(a) as being unpatentable over Midthun '85, Midthun 86, Hoshino *et al.* and Clark *et al.* in view of the above amendments and remarks.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance and an action to that end is respectfully requested. If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 206-467-9600.

Respectfully submitted,

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Serotype 1 reassortant of bovine rotavirus WC3, strain WI79-9, induces a polytypic antibody response in infants

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A reassortant rotavirus, strain WI79-9, was constructed bearing gene 9 of serotype 1 rotavirus strain WI79 and all other genes derived from bovine (serotype 6) rotavirus strain WC3. The antigenic phenotype of WI79-9 is bivalent: serotype 1 and serotype 6. WI79-9 administered orally at a dose of $10^{7.3}$ p.f.u. induced no adverse effects in 48 infants of age 2–11 months. Serotype 1- and serotype 6-specific serum neutralizing antibody titres were induced with approximately equal frequency in these infants. Serotype 1-specific antibody responses were inhibited in infants previously seropositive to type 1. The immune response rate was enhanced by administration of a second, 'booster dose'.

Keywords: Rotavirus; reassortant; WI79-9; vaccine

Introduction

We have previously reported that a strain of bovine-origin rotavirus, strain WC3, isolated in our laboratory is both safe and efficiently immunogenic in infants. A single dose of $10^{7.3}$ p.f.u. of WC3 at the 12th passage level induced a serum antibody response in 95% of infants 5–11 months of age in an initial trial of immunogenicity[1]. In a subsequent placebo-controlled double blind efficacy trial, a single dose of WC3 vaccine was associated with 76% protection against all rotavirus disease and 100% protection against moderate to severe rotavirus disease[2]. In each of these trials, adverse clinical reactions to vaccine were not detected.

Although these initial clinical studies yielded promising results in terms of immunogenicity, safety, and efficacy, WC3 vaccine is inefficient at inducing specific antibody to serotype 1 rotavirus. In recent years, serotype 1 has been the most common cause of infant disease world-wide[3–7]. Although partial heterotypic protection of infants was demonstrated in certain developed-nation situations with both WC3 vaccine[2] and bovine-origin rotavirus vaccine RIT 4237[8], protection induced by a primate origin rotavirus strain MMU18006 (RRV) usually appears to be type-limited[9–11]. It is possible that a vaccine with the desirable safety and immunogenicity characteristics of WC3 that is also capable of inducing type 1-specific antibody might exhibit improved performance in efficiency or duration of immune protection against natural rotavirus challenge. Therefore, we have constructed a reassortant rotavirus with type 1 antigenic phenotype added by means of incorporation of a single gene segment (gene segment 9 coding for surface protein vp7) of a type 1 rotavirus (strain WI79). The construction of this reassortant, strain WI79-9, and preliminary evaluation of its safety and immunogenicity in infants are described in this report.

Neither the virion structural protein localization of the antigen protective in active immunization against rotavirus disease nor the definitive need for serotype-specific antigenic determinants has been unequivocally demonstrated. Therefore, we have evaluated the immune response in terms of all neutralization antigen specificities known to be associated with WC3 and WI79 strain rotaviruses. Thus neutralization (PRN) antibody responses were assessed against WC3 (serotype 6) antigens associated with WC3 vp4 and WI79 (serotype 1) vp7 antigens retained in reassortant WI79-9. Antibody response to SA11 (serotype 3) was also assessed. WC3 administration has been associated with induction of PRN antibody to SA11 rotavirus in $\geq 50\%$ of infants[1,2]; serotype 3 rotavirus is a frequent cause of rotavirus disease world-wide[3].

Subjects, materials and methods

Cell culture

Continuous green monkey cell line MA104 was purchased from MA Bioproducts (Walkersville, MD, USA). Continuous green monkey cell line CV1[12] was obtained from the American Type Culture Collection (ATCC#CCL70). Cells were propagated in BHK medium as previously described[13].

Virus

The isolation of strain WC3 from a Pennsylvania calf with diarrhoea has been previously described[1]. Reference rotavirus strain SA11 (serotype 3)[14] was obtained from Dr Hubert Malherbe, San Antonio, TX, USA.

Strain WI79 rotavirus was isolated from the stool of a 5-month-old male infant with gastroenteritis admitted to Children's Hospital of Philadelphia on 20 March,

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1983. Rotavirus was isolated from the stool by methods previously described[15], modified from methods described by Sato *et al.*[16] and by Urasawa *et al.*[17]. The virus was identified as serotype 1 by Dr G. Gerna using the SPIEM technique[18] and by demonstration of selective neutralization by hyperimmune antisera to type 1 rotavirus and by serotype 1-specific monoclonal antibody 2C9[19]. All viruses were propagated and assayed for infectious titre by plaque assay as previously described[13].

Hyperimmune antisera to rotavirus were prepared by parenteral inoculation of guinea-pigs as previously described[1].

Subjects

Adult vaccinees were healthy young adults who gave informed consent. Volunteers were fed 30 ml of Maalox buffer followed immediately by vaccine. Clinical status was monitored by telephone contact for 7 days post-inoculation (p.i.). Stool and serum samples were taken immediately prior to vaccine administration. An additional stool sample was collected at day 3 p.i.; a serum sample was taken at 28 days p.i.

Infants

Infants aged 2–11 months were recruited at the outpatient clinics of the Children's Hospital of Philadelphia or at private paediatric practices in the Philadelphia suburbs. Informed consent was obtained from parents after a thorough explanation of the clinical trial protocol. Infants were free of congenital anomalies and acute or chronic illness, and had not received other immunizations (DPT or poliovirus) within 7 days of rotavirus inoculation. Nursing mothers were asked to refrain from feeding breast milk to their infants for 60 min before or after administration of vaccine. Most infants were given 30 ml of infant formula immediately prior to vaccine.

A physical examination was performed that included taking of rectal temperature. Vaccine was administered orally in two doses of 2.5 ml each separated by a 28 day interval. The study nurse contacted the parents daily for 7 days p.i.; during this period parents were instructed to record rectal temperatures twice daily in the morning and evening and to record the number and consistency of all stools.

Blood (0.5 ml) was collected by finger stick at the time of each inoculation and 28 days following the booster dose. Stool specimens (or rectal swabs) were collected at the time of administration of each vaccine dose and 3 days postadministration of each dose.

Laboratory evaluation of vaccine response

Sera were evaluated for titre of serum neutralizing antibody to rotavirus by the plaque-reduction neutralization (PRN) test as previously described[13]. A PRN antibody response was defined as: in seronegative infants, an increase in PRN titre to a given virus serotype of from $<1:50$ to $\geq 1:125$; or in the case of originally seropositive infants, an increase in PRN titre of at least three-fold over that observed prior to immunization.

Shedding of vaccine virus in faeces was evaluated by direct plaque assay of faecal suspensions on MA104 cell culture as previously described[1]. Plaques induced by faecal suspensions were propagated and analysed for the presence of the typical vaccine rotavirus RNA electropherotype by polyacrylamide electrophoresis[20].

Results

Development of reassortant WI79-9 vaccine

Reassortant WI79-9 was recovered from a mixed infection of MA104 cells with WC3 rotavirus (12th passage: candidate vaccine[1]) and WI79 rotavirus (24th passage: 11 passages in MA104 cells including two plaque-purifications, followed by 13 passages in CV1 cells). MA104 cells in a 24 well plastic plate (1 cm^2/well) were inoculated with WI79 virus (m.o.i. = 20.0) followed immediately by superinfection with WC3 virus (m.o.i. = 0.004). The whole cell culture was harvested when cytopathic effect involved the entire monolayer at 96 h postinfection, and was then reacted in a standard neutralization reaction with hyperimmune antiserum to serotype 6 bovine rotavirus at a dilution of 1:50. Virus surviving this neutralization reaction was titrated in a plaque assay; plaques were selected at random, propagated in MA104 cell culture, and analysed for RNA electropherotype by polyacrylamide gel electrophoresis[20]. A plaque-clone containing gene 9 of serotype 1 parent rotavirus WI79 with all other genes derived from WC3 rotavirus was selected for further study. This reassortant is designated as strain WI79-9.

The neutralization antigen phenotype of strain WI79-9 is illustrated in *Table 1*. Although hyperimmune guinea-pig sera to each parent virus of WI79-9 detected no cross-reaction with the heterotypic parent virus, reassortant WI79-9 is neutralized by antisera to either parent and is clearly antigenically bivalent. WI79-9 virus is neutralized by antiserum to WI79 virus to a titre identical to that observed with homologous WI79 virus. WI79-9 virus is neutralized by antiserum to WC3 rotavirus at a titre $\leq 5\%$ that of the homologous neutralization.

Reassortant WI79-9 was passaged six times in MA104 cells (including three plaque-clone purifications) followed by three passages in CV1 cell culture. The third passage in CV1 cell culture, with a titre of $10^{7.0}$ p.f.u. ml^{-1}, represents the test vaccine.

Inoculation of adults

A full dose ($10^{7.3}$ p.f.u.) of WI79-9 vaccine was administered to four adults between 25 and 45 years of age. No adverse clinical signs were noted. No adult

Table 1 Antigenic phenotype of reassortant WI79-9

Virus	Genotype	PRN antibody titre, antiserum to: WC3 (type 6)	WI79 (type 1)	Phenotype
WC3	Bovine (Type 6)	20905	<50	Type 6
WI79	Human (Type 1)	<50	4875	Type 1
WI79-9	Gene 9-WI79, all other genes = WC3	1070	5440	Type 6+Type 1

Table 2 Immune response of adults to WI79-9

Vaccinee	Age	Sex	Day p.i.	PRN antibody titre to serotype: 6 (WC3)	3 (SA11)	1 (WI79)
1	25	F	0	250	320	190
			28	920[a]	>1250[a]	1080[a]
2	44	M	0	390	175	750
			28	100	545[a]	965
3	39	F	0	>1250	>6250	5415
			28	>1250	>6250	5625
4	31	M	0	130	>1250	810
			28	920[a]	>1250	1200

[a]These titres represent a ⩾three-fold rise in serum PRN titre following administration of vaccine

Table 3 Infant response to a primary dose of WI79-9

No. of infants	Age	Virus dose	No. with symptoms	Faecal shedding of WI79-9	No. with PRN antibody response to: WC3	SA11	WI79	Any
2	12 months, 12 months	$10^{5.5}$	0	0/1	0	0	2	2
2	5 months, 11 months	$10^{6.5}$	0	0/1	0	0	1	1
25	2–4 months	$10^{7.5}$	0	1/24[b]	6	3	3	8 (32%)[a]
23	5–11 months	$10^{7.5}$	0	2/20[c]	14	10	14	19 (83%)[a]
2	12 months–3 years	$10^{7.5}$	1	1/2[d]	1	1	1	1
54			1	4/48 (8%)	21	14	21	31
All doses and ages:				39%	39%	26%	39%	57%

[a]Difference in response rate any serotype, age 2–4 months versus 5–11 months, $p = 0.0005$; [b]One infant shed WI79-9 at a concentration of $10^{2.6}$ p.f.u. g^{-1} faeces. Ten infants shed vaccine poliovirus. One infant shed an enterovirus (untyped); [c]Two infants each shed WI79-9 at a concentration of $10^{2.3}$ p.f.u. g^{-1}, one infant shed enterovirus (untyped); [d]The 12-month-old infant shed WI79-9 virus at a concentration of $10^{4.9}$ p.f.u. g^{-1}; this infant did not exhibit an SN antibody response; a fever on day 3 and day 4 p.i. (39.2 C and 39 C, respectively) was not accompanied by symptoms of gastroenteritis

showed detectable WI79-9 virus in stool at 3 days p.i. Serum PRN antibody responses are shown in *Table 2*. Although each volunteer was seropositive (titre >100) prior to immunization to both of the serotypes 6 and 1 represented on the WI79-9 reassortant, positive PRN antibody responses to one or more serotypes were noted in three of four vaccinees. The only vaccinee who did not exhibit a PRN antibody response exhibited serum PRN titres of >1:1250 to serotypes 1, 6 and 3 prior to receiving the vaccine.

Inoculation of infants: primary dose

The response of infants to a primary dose of WI79-9 is presented in *Table 3*. After initial inoculations of two infants, each with vaccine at a dilution of 10^{-2} ($10^{5.3}$ p.f.u.) and 10^{-1} ($10^{6.3}$ p.f.u.), respectively, caused no adverse effects, a total of 48 infants aged 2–11 months, one 12-month-old infant and one 3-year-old child were given a full WI79-9 dose of $10^{7.3}$ p.f.u. No evidence was observed of WI79-9 vaccine-associated clinical symptoms in any vaccinee (under the age of 12 months). Shedding of WI79-9 strain rotavirus was evaluated by plaque assay of stools collected 3 days p.i. in 48 infants given a full dose; WI79-9 virus was recovered from four (8%). Three of the four shed WI79-9 at a concentration of less than $10^{3.0}$ p.f.u. g^{-1} of faeces. One infant inoculated at age 12 months shed WI79-9 at a concentration of $10^{4.9}$ p.f.u. g^{-1} of faeces and exhibited a transient fever on days 3 and 4 p.i. (maximum temperature was 39.4 C).

The efficiency of the rotavirus-specific serum-neutralizing antibody response was clearly age-dependent. Eight of 25 infants (32%) inoculated at age 2–4 months exhibited a serum antibody response whereas 19 of 23 (83%) infants inoculated at age 5–11 months exhibited an immune response. In both age groups, neutralizing antibody responses were induced to bovine WC3 (serotype 6), WI79 (serotype 1) and SA11 (serotype 3) rotavirus. Immune responses were directed most often and with equal frequency to serotype 6 and serotype 1 rotavirus (each response observed in 39% of all vaccinees) and less frequently to serotype 3 rotavirus).

Effect of pre-immunization serum antibody

The incidence and specificity of the immune response to WI79-9 were analysed in relation to the prevalence of prevaccination serum antibody (PRN antibody titre ⩾1:100) in the vaccinees (*Table 4*). The percentage of infants with pre-existing antibody to each of the three serotypes evaluated was higher in the 2–4-month-old group than in the 5–11-month-old infants. However, pre-existing serum antibody did not influence the immune response to WI79-9 in the younger group. On the contrary, in the 5–11-month-old group pre-existing antibody to either of the major serotype antigens represented on the WI79-9 virion, serotype 6 or serotype 1, was associated with inhibition of the serum PRN antibody response to a statistically significant degree. In particular, prior antibody to type 6 or to type 1 was specifically associated with inhibition of the homotypic active immune response to type 6 or to type 1, respectively.

Other factors

Forty-four percent of 2–4-month-old infants and 17% of 5–11-month-old infants were breast fed at the time of

immunization. Breast feeding was not associated with inhibition of immune response in either group.

Buffer (infant formula) was administered prior to vaccine in 64% of 2–4-month-old infants and 61% of 5–11-month-old infants. Immune response rates were slightly higher in the buffered infants in each age group (2–4 months, 38% buffer versus 22% no buffer; 5–11 months, 93% buffer versus 67% no buffer) but differences were not statistically significant.

Immune response rates were not affected by sex, race, or the clinical site of vaccination.

Inoculation of infants: booster dose

Studies with WC3 rotavirus vaccine have indicated that a second booster dose at 28 days p.i. enhanced the response rate particularly in 2–4-month-old infants and also caused an increased incidence of heterotypic antibody responses to serotype 3 strain SA11 (H.F. Clark, F. Borian, and S.A. Plotkin, manuscript in preparation). Therefore, we administered a booster dose of vaccine to 44 infants at 28 days after an initial inoculation of WI79-9. A total of 33 infants were given WI79-9 as a booster dose while 11 infants were given a booster dose of WC3 rotavirus vaccine of identical infectious titre. The serum PRN antibody responses to a booster dose of WI79-9 are illustrated in *Table 5*.

In contrast to results of the primary inoculation, the rate of immune response to a second dose of WI79-9 was equal or slightly higher in the younger group (2–4 months old at primary immunization) as compared with the older group of infants. As a result, 59% of the younger infants and 88% of the older infants exhibited an immune response after two doses of WI79-9. The serotype-specificity of the booster response was of approximately equal frequency to serotypes 6, 3 and 1 in the 2–4-month-old infants but was most frequently directed to serotype 6 in the older infants. After two doses, the combined response was detected with approximately equal frequency to each of the three serotypes tested in each age group.

A small number of infants (11) were given a booster dose of WC3 vaccine (*Table 6*). By chance, this group exhibited an unusually high incidence of positive primary responses to WI79-9, especially in the 2–4-month-old infants. This group again demonstrated booster immune responses at rates similar to those observed with a booster dose of WI79-9.

Effect of immune state after dose 1 on booster response to WI79-9

We analysed the effect of the rotavirus immune state of the infant host after an initial inoculation of WI79-9 upon the response to a booster dose of WI79-9, with regard to the influence of prior seropositivity (titre $\geq$1:100) and an active immune response to the initial dose. Infants who were seropositive to type 6 or type 3

Table 4 Effect of pre-immunization antibody on primary response to WI79-9 rotavirus

Serotype	Pre-antibody status	Age (months)	No. of infants	No. (%) with PRN antibody response to serotype: Any	6 (WC3)	3 (SA11)	1 (WI79)
Any	–	2–4	19	7 (37)			
	–	2–4	6	1 (17)			
	+	5–11	10	6 (60)*			
	–	5–11	13	13 (100)*			
6 (WC3)	+	2–4	9	4 (44)	2 (22)		
	–	2–4	16	4 (25)	4 (25)		
	+	5–11	7	4 (57)	2 (29)*		
	–	5–11	16	15 (94)*	12 (75)*		
3 (SA11)	+	2–4	9	2 (22)		1 (11)	
	–	2–4	16	6 (38)*		2 (13)	
	+	5–11	4	3 (75)*		2 (50)*	
	–	5–11	19	16 (84)*		8 (42)*	
1 (WI79)	+	2–4	14	4 (29)*			0 (0)
	–	2–4	11	4 (36)*			3 (27)
	+	5–11	5	1 (20)*			1 (20)*
	–	5–11	18	18 (100)*			13 (72)*

*$p \leq 0.05$

Table 5 Immune response of infants to primary and booster inoculations of WI79-9

Dose	No. of infants	Age* (months)	Percentage PRN antibody response to: WC3 (6)	SA11 (3)	WI79 (1)	Any
Primary	17	2–4	12	6	0	18
Booster†	17	2–4	41	47	35	53
Combined‡	17	2–4	47	47	35	59
Primary	16	5–11	69	44	63	75
Booster	16	5–11	38	25	12	44
Combined	16	5–11	81	62	75	88

*Age of primary dose inoculation. †A booster response is defined as development of a titre of $\geq$1:125 in an infant with a titre of <1:50 after the primary dose, or development of a $\geq$3-fold increase in the titre observed after the primary dose. ‡Response to primary dose or secondary dose or to both doses

Table 6 Immune response of infants to primary inoculation with WI79-9 and booster inoculation with WC3

Dose	No. of infants	Age[a] (months)	Percentage PRN antibody response to:			
			WC3 (6)	SA11 (3)	WI79 (1)	Any
Primary	7	2–4	57	29	43	71
Booster[b]	7	2–4	57	29	14	71
Combined[c]	7	2–4	86	57	43	100
Primary	4	5–11	75	0	50	100
Booster	4	5–11	100	100	75	100
Combined	4	5–11	100	100	100	100

[a]Age at primary inoculation; [b]A booster response is defined as development of a titre of $\geq$1:125 in an infant with a titre of <1:50 after the primary dose, or development of a $\geq$3-fold increase in the titre observed after the primary dose; [c]Response to primary dose or secondary dose or to both doses

showed reduced incidence of booster response to types 6, 3 or 1, but these trends did not consistently reach the level of statistical significance. Infants seropositive to type 1 exhibited no depression of booster immune response to types 6, 3 or 1.

In contrast, although an active response to serotype 1, 3 or 6 after the first dose was not associated with inhibition of an active booster response to type 6 or 3, it was associated with marked inhibition of a booster response to type 1 [0 of 10 infants (0%) with a primary type 1 response exhibited a booster type 1 response, compared with 8 of 23 infants (35%) who exhibited no type 1 primary response but did develop a booster type 1 response ($p=0.03$)]. In other words, infants who responded to a primary inoculation with a type 6 or type 3 PRN antibody response had the same chance of responding to an identical booster dose with a further increased antibody titre, whereas a primary dose response to type 1 appeared to block a second response to type 1 when booster was administered.

As in the case of primary dose responses, the incidence of booster dose immune response was not affected by breast feeding but was relatively elevated in infants administered buffer prior to vaccine (data not shown). Two of 34 infants tested for faecal shedding exhibited WI79-9 vaccine virus in stool after boost; both of these infants had exhibited an active serum antibody immune response to the primary inoculation. No adverse clinical effects were detected after booster doses of either WI79-9 or WC3 vaccines in infants 2–11 months of age. The infant 12 months of age at initial inoculation with WI79-9 exhibited a fever of 38.1°C on the evening of day 0 post-boost only, but did not shed detectable virus in faeces after the boost.

Discussion

Reassortant WI79-9, in which gene 9 (coding for vp7) of a human serotype 1 isolate WI79 was added to a genome background of serotype 6 strain WC3 virus, acquired a bivalent type 1 and type 6 neutralization antigen phenotype and retained the excellent safety characteristics of WC3 rotaviruses. WI79-9 induced no disease symptoms in four adult volunteers and none shed virus in stool. None of 52 infants 2–11 months of age exhibited adverse reactions to vaccine and faecal shedding of vaccine virus was detected in only 3/44 (6.8%) of those evaluated after a full $10^{7.3}$ p.f.u. dose. This shedding rate is similar to that detected in two previous trials of WC3 vaccine (21%[1] and 5%[2], respectively). A single 12-month-old infant shed vaccine virus at a concentration ($10^{4.9}$ p.f.u. g^{-1}) higher than that previously observed with WC3 and exhibited a transient fever (maximum temperature = 39.4°C) on days 3 and 4 after inoculation. This infant was seronegative to type 1, 3 and 6 rotavirus prior to inoculation and did not exhibit a serum antibody response to the primary or to a booster dose of WI79-9 vaccine. This anomalous host response cannot presently be explained.

The immune response rate (any serum PRN antibody response to type 1, 3 or 6 rotavirus) was, as previously noted with WC3 vaccine, markedly less in 2–4-month-old infants (32%) than in 5–11-month-old infants (83%). Such response rates were similar to the WC3 response rates of 44% noted in 2–4-month-old infants (H.F. Clark, F.E. Borian, K. Modesto, and S.A. Plotkin, unpublished data), and, in two different studies, 95%[1] or 71%[2] in 5–11-month-old infants. The reduced capacity of 2–4-month-old infants to respond to rotavirus vaccines is unexplained, but may be related to higher levels of virus-specific natural immunity in these younger infants and/or to a delayed maturation of the infant immune system.

There are two major surface structural proteins of rotavirus, vp4 and vp7, each of which is capable of eliciting a virus-neutralizing antibody response[21–23]. As the WI79-9 reassortant rotavirus contains vp4 and vp7 components of different and completely antigenically distinct parents (in terms of neutralization antigens), it is interesting to evaluate the infant immune response in terms of the separate type 1 (vp7, WI79) and type 6 (vp4, WC3) neutralizing antibody responses. Although the virion structural localization of the antigen eliciting antibody to SA11 (type 3) rotavirus has not been determined, serotype cross-reactive antigens on other rotavirus strains have been most often associated with vp4[15,24].

The infant immune response to serotype 1 rotavirus induced by reassortant WI79-9 was clearly enhanced when compared with WC3. The immune response rate to type 1 in all age groups following a single dose of the reassortant was 39%, which was equal to the response rate to serotype 6 (WC3). This compares with serotype 1 serum antibody response rates of <10% observed in two different studies of WC3[1,2].

In contrast with observations reported with the non-human (serotype 6) WC3 vaccine, the infant response rate to WI79-9 was demonstrably affected negatively by the pre-existing immune state of the host. Inhibition of immune response associated with prior immunity was seen only in 5–11-month-old infants. This may indicate that an active immune response in these

older infants is inhibitory while the presence of antibody passively acquired from the mother is not. In the older infants, inhibition of any antibody response was most marked in those with antibody to serotype 1, less prominent in those seropositive to serotype 6, and absent in those seropositive to serotype 3. The serotype 6 response was selectively inhibited in infants seropositive to serotype 6.

The serotype 1 response was selectively inhibited in 2–4-month-old infants seropositive to type 1, despite the fact that the response of such infants to 'any serotype (1, 3, or 6)' was not. Therefore, although the serotype 1 reassortant WI79-9 clearly exhibits an enhanced capacity (compared to WC3) to induce a serotype 1-specific immune response, this benefit is expressed only in infants seronegative to type 1 prior to immunization.

In contrast to the result of a primary immunization with WI79-9 reassortant, a booster dose was approximately equally effective (no statistically significant difference) in infants of either age group studied, thereby yielding final response rates of 59% in infants originally 2–4 months old and 88% in those originally 5–11 months old. The inhibition of immune response to an initial dose selectively associated with seropositivity to rotavirus type 1 was not exhibited with a booster dose, indicating that a second dose is useful in overcoming prevaccine immunity. On the other hand, a primary dose response to type 1 was associated with failure to mount a booster dose to type 1, whereas primary responses to type 6 or 3 were not associated with inhibition of booster response. Thus the results suggest that sequentially both primary and booster responses may be elicited to antigenic determinants of the same serotype on vp4 (not necessarily the same epitopes) by two successive rotavirus inoculations, but a primary immune response to the serotype-specific determinants on vp7 may render a booster response to vp7 determinants unlikely. Analysis of other reassortants will be necessary to see if this divergent pattern of booster responses to vp4 and 7 is a general phenomenon or particular to the antigens of reassortant WI79-9.

Analysis of the response to one or two doses of WI79-9 clearly indicates that the type 1 response rate is enhanced when compared with that previously reported with rotavirus WC3 alone ($p=0.026$). This observation appears to justify further evaluation of reassortant WI79-9 for protective efficacy against natural rotavirus challenge, which is most frequently associated with serotype 1. A preliminary efficacy trial in which WI79-9 immunization was associated with protection against challenge, during a season when natural infection was caused by both type 1 and type 3 viruses, will be reported elsewhere[25].

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